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APPLICATION NUMBER: *60/458,224*
FILING DATE: *March 27, 2003*
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03-28-03

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ~~EV 243854760 US~~ EU907681047US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (city and either State or Foreign Country)
Jeffrey A.	WHITSETT	5565 Salem Road, Cincinnati, OH 45230

[ ] *Additional Inventors are being named on the __ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

USE OF FGF-18 PROTEIN AND ITS RESPECTIVE ENCODING NUCLEOTIDE SEQUENCES TO INDUCE CARTILAGE FORMATION

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification - *Number of Pages* 30 [ ] CD(s), Number [ ]

[X] Drawing(s) - *Number of Sheets* 9 [X] Other (specify) sequence Listing (4 pages)

[ ] Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant claims small entity status. See 37 CFR 1.27
[ ] A check or money order is enclosed to cover the filing fees
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[X] Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($)

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Eric W. Guttag

DATE: March 27, 2003

REGISTRATION NO. 28,853

TYPED or PRINTED NAME Eric W. Guttag

DOCKET NUMBER CHM-003PAT-P1

TELEPHONE 513-229-0383

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Created 02/2003
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FEE TRANSMITTAL

(Patent Fees are subject to annual revision)

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	J. A. Whitsett
Examiner Name	
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[X] Applicant claims small entity status. (See 37 CFR 1.27)

TOTAL AMOUNT OF PAYMENT $80.00

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[ ] Check [X] Credit Card [ ] Money Order [ ] Other [ ] None

[ ] Deposit Account:

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The Commissioner is authorized to: *(check all that apply)*

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[ ] Charge any additional fee(s) during the pendency of this application

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Small Entity Fee Code	Fee Description	Fee Paid
1001	2001	Utility filing fee	.00
1002	2002	Design filing fee	.00
1003	2003	Plant filing fee	.00
1004	2004	Reissue filing fee	.00
1005	2005	Provisional filing fee	80.00

Subtotal (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from Below		Fee Paid
Total Claims	[ ] - 20** =	[ ]	X	[ ]	=	.00
Independent Claims	[ ] - 3** =	[ ]	X	[ ]	=	.00
Multiple Claims				[ ]	=	.00

Large Entity Fee Code	Small Entity Fee Code	Fee Description
1202	2202	Claims in excess of 20
1201	2201	Independent claims in excess of 3
1203	2203	Multiple dependent claim, if not paid
1204	2204	**Reissue independent claims over original patent
1205	2205	**Reissue claims in excess of 20 and over original patent

Subtotal (2) ($) 0.00

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Codes	Small Entity Fee Codes	Fee Description	Paid
1051	2051	Surcharge – late filing fee or oath	[ ]
1052	2052	Surcharge – late provisional filing fee or cover sheet	[ ]
1053	1053	Non-English specification	[ ]
1812	1812	Filing a request for ex parte reexamination	[ ]
1804	1804	* Requesting publication of SIR prior to Examiner action	[ ]
1805	1805	Requesting publication of SIR after Examiner action	[ ]
1251	2251	Extension for reply within first month	[ ]
1252	2252	Extension for reply within second month	[ ]
1253	2253	Extension for reply within third month	[ ]
1254	2254	Extension for reply within fourth month	[ ]
1255	2255	Extension for reply within fifth month	[ ]
1401	2401	Notice of Appeal	[ ]
1402	2402	Filing a brief in support of an appeal	[ ]
1403	2403	Request for oral hearing	[ ]
1451	1451	Petition to institute a public use proceeding	[ ]
1452	2452	Petition to revive – unavoidable	[ ]
1453	2453	Petition to revive - unintentional	[ ]
1501	2501	Utility issue fee (or reissue)	[ ]
1502	2502	Design issue fee	[ ]
1503	2503	Plant issue fee	[ ]
1460	1460	Petitions to the Commissioner	[ ]
1807	1807	Processing fee under 37 CFR 1.17(q)	[ ]
1806	1806	Submission of Inform. Discl. Statement	[ ]
8021	8021	Recording each patent assignment	[ ]
1809	2809	Filing a submission after final rejection (37 CFR 1.29(a))	[ ]
1810	2810	For each additional invention to be examined (37 CFR 1.129(b))	[ ]
1801	2801	Request for Continued Examination (RCE)	[ ]
1802	1802	Request for expedited examination of a design application	[ ]
Other fee (specify) ___			[ ]

*Reduced by Basic Filing Fee Paid

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SUBMITTED BY				Complete (if applicable)	
Name (print/type)	Eric W. Guttag	Registration No.	28,853	Telephone	513-229-0383
Signature	Eric W. Guttag			Date	March 27, 2003

Created 2/2003
PTO/SB/17 (01-03)

USE OF FGF-18 PROTEIN AND ITS RESPECTIVE ENCODING NUCLEOTIDE SEQUENCES TO INDUCE CARTILAGE FORMATION

Jeffrey A. Whitsett

TECHNICAL FIELD

[0001] The present application relates to fibroblast growth factor (FGF)-18 protein, the respective nucleotide sequences encoding this protein, and their use in inducing cartilage formation, particularly for the purpose of generating, repairing, reconstructing, or *de novo* formation of, cartilaginous tissue. The present application further relates to the use of compositions containing FGF-18 protein for inducing such cartilage formation, or the respective gene encoding this protein to induce such formation.

BACKGROUND OF THE INVENTION

[0002] Cartilage is a specialized type of dense connective tissue consisting of cells embedded in a matrix. There are several kinds of cartilage. Translucent cartilage having a homogeneous matrix containing collagenous fibers is found in articular cartilage, in costal cartilages, in the septum of the nose, in the larynx and in the trachea. Articular cartilage is hyaline cartilage covering the articular surfaces of bones, while costal cartilage connects the true ribs and the sternum. Yellow cartilage is a network of elastic fibers holding cartilage cells which is primarily found in the epiglottis, the external ear, and the auditory tube. See U.S. Patent 6,258,778 (Rogers et al), issued July 10, 2001.

[0003] Cartilage tissue is made up of an extracellular matrix primarily comprised of collagens, glycosaminoglycans, and proteoglycans, along with chondrocyte cells, which are synthesized and secrete these components that assemble into cartilage. These components, and the water entrapped within these organic matrix elements, yield the unique elastic properties and strength of cartilage. Wozney et al, *Science*, (1988) 242:1528-1533; Sporn et al, *J. Cell. Biol.*, (1987) 105:1039-1045. See also U.S. Patent 6,258,778 (Rogers et al), issued July 10, 2001 Morphogenesis of cartilage is of fundamental importance in that development of this tissue underlies and determines the form of much of the endoskeleton of vertebrates. Cartilage is the dominant skeletal material of early embryonic life, and becomes a permanent part of craniofacial, auricular, laryngeal, costal, and articular structures. Junctions between skeletal elements are almost entirely cartilaginous, and it is the precise morphogenesis of

articular surfaces that ensures normal joint development and efficient joint function. See Tickle et al, *Curr. Opin. Genet. Dev.*, (1995) 5:478-84; Johnson et al, *Cell.*, (1997) 90:979-90.

[0004] Like skeletal cartilage, the cartilage structures associated with the respiratory system arise from primitive structures of embryonic cartilage. The trachea forms as an outpouching of the endoderm shortly after it fuses into the embryonic endodermal gut tube. The primitive larynx is derived from the second and fourth pharengeal arches, caudal to the tracheal outpouch. Endodermal epithelial cells of the outpouch and its primary and secondary branches invade the surrounding mesoderm, initiating a process known as branching morphogenesis. Interactions of the proximal endoderm with the surrounding mesoderm result in chondrogenesis, and formation of a series of cartilage ring structures ensues. These cartilaginous rings provide flexible support that contributes to maintenance of airways after birth. The signals inducing formation of cartilage are extinguished or suppressed in the distal endoderm, thus the developing lung parenchyma remains essentially devoid of cartilage. See Larsen, *Human Embryology*, (Churchill Livingstone, Inc., NY, 1993).

[0005] In general, disturbances in the control of cartilage morphogenesis can result in dysmorphogenesis of the skeleton, larynx, trachea, ear, nose, and other cartilaginous tissues, and such an event usually has deleterious effects. While some such types of dysmorphogenesis can be lethal, others can result in devastating deformities that are difficult or impossible to remedy with reconstructive surgery. The relatively poor vascularization of cartilage is also a serious obstacle in the healing process required for recovery from any type of plastic surgery. In addition, injury to cartilage can cause serious morbidity to this tissue. Since cartilage tissue is generally resistant to healing, the extended period of time required for healing frequently enables recurrent damage and compromises recovery from cartilage injuries. See *Cartilage* Vol. 1-3 (B.K. Hall, Ed.; Academic Press, NY, 1983).

[0006] Abnormalities of cartilage formation and development are major medical problems in several diseases. Likewise, cartilage damage and degeneration are associated with many clinical conditions of serious consequence. These cartilage-related disorders account for nearly $2.5 billion in health care cost per year worldwide. Current therapies focus on reducing inflammation, pain management and increasing range of motion where joints are involved. However, therapies and treatments directed at inhibiting cartilage degeneration or, more importantly, promoting the formation, growth and development of cartilage remain

elusive.

[0007] Articular cartilage has recently been demonstrated to have a spontaneous repair response in the case of full-thickness cartilage defects. See Shapiro et al, *J. Bone Jt. Surg.*, (1993) 75-A(4):532-53. However, this repair response is limited in terms of form and function. By contrast, there have been no meaningful repair processes in partial-thickness lesions limited to the cartilage itself. See Hunziker et al, *J. Bone Jt. Surg.*, (1996) 78-A:721-33. While many repair techniques have been proposed over the past four decades, none have successfully regenerated long-lasting hyaline cartilage tissue to replace damaged cartilage. See Buckwalter et al, *J. Bone Jt. Surg.*, (1997) 79-A:612-32. In fact, most of the surgical interventions to repair damaged cartilage have been directed toward the treatment of clinical symptoms, such as pain relief and functional restoration of joint structures and articulating surfaces, rather than regeneration of hyaline cartilage. See Johnson et al. *Arthroscopy*, (1989) 2:54-69.

[0008] An initial surgical attempt to restore the normal articulating surface of joint cartilage has been made with the introduction of Pridie's resurfacing technique. This chondral repair technique utilizes the disruption of subchondral bone to induce bleeding from the bone marrow, thus promoting the regular wound-healing mechanism in the cartilage defect site. See Pridie, "Proceedings of the British Orthopaedic Association," *J. Bone Jt. Surg.*, (1959) 41B:618-19. Since Pridie's abrasion arthroplasty, several subchondral disruption techniques have been introduced in an attempt to improve the healing mechanisms of repaired tissue. These include subchondral drilling, arthroscopic abrasion, and microfracture techniques. See Mitchell et al, *J. Bone Jt. Surg.*, (1976) 58A(2):230-33; Brown et al, *J. Sports Med.*, (1974) 2:27-46; Bert et al, *Rheum. Dis. ClinNAm.*, (1993) 19:725-39; Rodrigo et al, *Am. J. Knee Surg.*, (1994) 7(3):109-16.

[0009] Several experimental animal studies on full-thickness cartilage repair have also revealed that subchondral breaching techniques create a fibrin clot formed from bleeding in the region of the cartilage defect. This clot is subsequently infiltrated by mesenchymal cells, and acts as a three-dimensional scaffold for migrating progenitor cells. See Shapiro et al, *J. Bone Jt. Surg.*, (1993) 75-A(4):532-53. Within six weeks, these cells gradually differentiate and completely fill the defect region with a hyaline-like repair cartilage. However, this newly synthesized repair cartilage typically contains significantly less proteoglycan than normal hyaline cartilage. See Mitchell et al, *J. Bone Jt. Surg.*, (1976) 58A(2):230-33. The repair tissue at this stage is also more cellular than the adjacent normal

cartilage and shows no structural integration with the residual cartilage. As a result, there will be degeneration and fissuring in the cartilage tissue. While the full-thickness defect spontaneous repair mechanism is present for the first several weeks after surgical repair, it later fails due to inadequate mechanical and biochemical conditions in the repaired tissue. See O'Driscoll et al, *J. Bone Jt. Surg.*, (1988) 70-A(4):595-606; Scherping et al, *American Orthopaedic Society for Sports Medicine*, (Toronto, ONT, Canada, 1995); Suh et al, *Oper. Tech. Orthop.*, (1997) 7:270-78.

[0010] The concept of tissue engineering as an approach to cartilage repair was first proposed by Green in 1977. See Green et al, *Clin. Orthop. Relat. Res.*, (1977) 124:237-50. In this approach, chondrocytes grown in an *ex vivo* environment are transplanted into the cartilage defect. Clinical application of such a tissue engineering approach was first attempted by a Swedish group. See Brittberg et al, *New Engl. J. Med.*, (1994) 331(14):889-95. Chondral repair techniques utilizing laboratory-grown cells have attracted significant attention. See Buckwalter et al, *J. Bone Jt. Surg.*, (1997) 79-A:612-32. Recently, tissue engineering concepts have been introduced to develop cell-based repair approaches for articular cartilage. See Freed et al, *J. Biomed. Mater. Res.*, (1993) 27:11-23; Vacanti et al, *AJSM*, (1994) 22(4):485-88. Tissue engineering of articular cartilage involves the isolation of articular chondrocytes or their precursor cells that can be expanded *in vitro* and then seeded into a biocompatible matrix, or scaffold, for cultivation and subsequent implantation into the joint. The type of cell used to engineer cartilage is critical to the long-term outcome. Different cell populations that have been investigated in the experimental studies include matured articular chondrocytes, epiphyseal chondrocytes, mesenchymal stem cells, bone marrow stromal cells, and perichondrocytes. See Robinson et al, *Methods in Cartilage Research*, (London: Academic Press, 1990), pp. 327-30; Bently et al, *Nature*, (1971) 230:385-88; Itay et al, *Clin. Orthop. Relat. Res.*, (1987) 220:284-301; Wakitani et al, *J. Bone Jt. Surg.*, 1989) 71-B:74-80; Butnariu-Ephrat et al, *Clin. Orthop. Relat. Res.*, (1996) 330:234-43; Martin et al, *J. Orthop. Res.*, (199) 16:181-89; Wakitani et al, *J. Bone Jt. Surg.*, (1994) 76-A(4):579-92; Chu et al, *J. Biomed. Mater. Res.*, (1995) 29:1147-54.

[0011] The choice of biomaterial is also critical to the success of such tissue engineering approaches in cartilage repair. A variety of biomaterials, naturally occurring and synthetic, biodegradable and non-biodegradable, have been used as potential cell-carrier substances for cartilage repair. See Grande et al, *J. Biomed. Mater. Res.*, (1997) 34:211-20. The naturally occurring biomaterials include various forms of types I and II collagen-based

biomaterial in the form of scaffold matrices, gels, or collagen/alginate composite gels. See Speer et al, *Clin. Orthop. Relat. Res.*, (1979) 144:326-35; Sams et al, *OA. Cartilage*, (1995) 3:47-59; Frenkel et al, *J. Bone Jt. Surg.*, (1997) 79-B:831-36; Nehrer et al, *J. Biomed. Mater. Res.*, (1997) 38:95-104; Kang et al, *OA. Cartilage*, (1997) 5:139-43; Kimura et al, *Clin. Orthop. Relat. Res.*, (1984) 186:231-39; Qi et al, *J. Orthop. Res.*, (1997) 15(4):483-90. The synthetic polymer-based biomaterials include polyglycolic acid (PGA) and poly-L-lactic acid (PLLA), or their composite mixture. In cartilage tissue engineering, PGA, PLLA, and PGA/PLLA copolymers have been studied for their efficacy as chondrocyte-delivering scaffolds in vitro and in vivo. See Freed et al, *J. Biomed. Mater. Res.* (1994) 28:891-99; Woo et al, *Plastic Reconstr. Surg.*, (1994) 94(2):233-37; Athanasiou et al, *Biomaterials*, (1996) 17:93-102. Several investigators have also found that some non-biodegradable polymer substances, such as polytetrafluoroethylene, polyethylmethacrylate, and hydroxyapatite/Dacron composites, also facilitate the restoration of an articular surface. Reisses et al, *Transactions of the Orthopaedics Research Society*, (New Orleans, LA, 1994); Messner et al, *Biomaterials*, (1993) 14:513-21. The ideal cell-carrier substance is one that most closely mimics the naturally occurring environment in the articular cartilage matrix.

[0012] It has been shown that cartilage-specific extracellular matrix components such as type II collagen and glycosaminoglycan (GAG) can play a critical role in regulating expression of the chondrocytic phenotype and in supporting chondrogenesis both *in vitro* and *in vivo*. See Kosher et al, *Dev. Biol.*, (1973) 35(2):210-20; Kosher et al, *Nature*, (1975) 258:327-30. Otherwise, chondrocytes can undergo de-differentiation and produce an inferior fibrocartilaginous matrix rich in type I collagen. This inferior matrix can then lead to a failure to form hyaline cartilage. See von Der Mark et al, *Nature*, (1977) 267:531-32; Sokoloff et al, *J. Rheumatol.*, (1974) 1:1-10. Thus, the criteria for the choice of biomaterial in cartilage tissue engineering include biological friendliness and biomechanical strength. These features can provide a biochemically and biomechanically appropriate environment necessary for engineered cells to regenerate a long-lasting hyaline cartilage in the defect site. See Langer et al, *Transactions of the 44th ORS*, (New Orleans, 1998).

[0013] Also crucial to the tissue engineering process is the identification of growth factors that induce chondrogenesis. Many growth factors have been found to play a role in regulating cartilage development, remodeling or repair. For instance, it has previously been shown that the endogenous growth factors TGF-beta and various BMP family members induce both new cartilage and bone formation. However, specific signals that induce the

formation of cartilage have previously remained unknown.

[0014] Restoration of epithelial tissue, such as tracheal tissues, after tissue injury is a complex process, which includes several critical events, including deposition of the extracellular matrix, tissue remodeling, and angiogenesis. These events are coordinated with epithelial cell migration and proliferation to restore the epithelial and/or mucosal barrier (i.e., in epithelial tissues such as tracheal epithelium which secrete mucous). The coordination of these events is believed to involve the interaction between different classes of cells as well as between cells and their extracellular matrix. See U.S. Patent 6,465,205 (Hicks), issued October 15, 2002.

[0015] Present approaches to tracheal repair include resection and reanastomosing the injured airway, replacement of the damaged portion by synthetic material, and use of autologous tissue for reconstruction of the tracheal defect. See Letang et al., "Experimental Reconstruction of the Canine Trachea with a Free Revascularized Small Bowel Graft," *Ann. Thorac. Surg.*, (1990) 49:955-58; Mulliken et al, "The Limits of Tracheal Resection with Primary Anastomosis: Further Anatomical Studies in Man," *J. Thorac. Cardiovasc. Surg.*, (1968) 55:418 (abstract); Neville et al., "Prosthetic Reconstruction of the Trachea and Carina," *J. Thorac. Cardiovasc. Surg.*, (1976) 72:525-36. Recently, tissue engineering approaches have been used in such repair, including forming an *in vivo* tracheal cartilaginous scaffolding by injecting dissociated chondrocytes into a preformed synthetic construct. See Hirano et al., "Hydroxylapatite for Laryngotracheal Framework Construction," *Ann. Otol. Rhinol. Laryngol.*, (1989) 98:713-17; Okumura et al., "Experimental Study of a New Tracheal Prosthesis Made from Collagen Grafted Mesh," *Trans. Am. Soc. Artif. Organs.*, (1991) 37:M317-19; Langer et al., "Tissue Engineering," *Science*, (1993) 260:920-26.

[0016] However, there is still a need for therapies to generate cartilage and to treat various abnormalities or injuries that occur in various cartilaginous tissues, including of those of the joints, eye and nose and especially the cartilaginous tissues of various connecting airways such as the trachea, bronchi, lung and larynx: In particular, there is a need to develop effective cartilage generation, repair and reconstruction techniques for abnormalities or injuries that occur in such cartilaginous tissues.

BRIEF DESCRIPTION OF THE INVENTION

[0017] The present invention relates to the discovery that fibroblast growth factor (FGF)-18 protein is capable of inducing cartilage formation, as well as the use of nucleotides

sequence(s) encoding FGE-18 protein for various purposes, including generation, repair, reconstruction, *de novo* formation or other formation of a variety of cartilaginous tissues. In particular, selective regulation of FGF-18 can induce cartilage programming during development of tissues, including tracheal-bronchial cartilage tissue formation in the conducting airways. FGF-18 protein and its receptor and signaling pathways can be used to induce new cartilage formation or expand cartilage growth in various sites of the body, including the tracheal-bronchial rings of the conducting airways and larynx, as well as other sites where cartilage deposition would be therapeutic or beneficial such as the cornea, nose, ear, ribs, sternum, joints and bones. Therapies for which FGF-18 are useful include repair and reconstruction of various tissues in conducting airways such as the trachea, bronchi, lung and larynx caused by, for example, congenital or pathological tracheal-bronchial abnormalities. Other therapies for which FGF-18 would be useful include other cartilaginous tissues, such as those of joint and skeletal tissue caused by, for example, arthritis and meniscus abnormalities in joints.

[0018] It has been found that FGF-18 provides an appropriate induction signal to mesenchymal cells that results in chondrogenesis and subsequent formation of cartilage. For example, mice with ectopic expression of FGF-18 in pulmonary epithelial cells have reproducible ectopic cartilage formation and expansion of cartilage at normal bronchial sites in the lung. These cartilage cells have been demonstrated histologically by alcion blue staining in ectopic regions in the lung, and by immunohistochemical staining for collagen type II, a marker of early cartilage differentiation and expansion. By contrast, ectopic expression of FGF-7 or FGF-10 does not reproduce the changes in cartilage observed in mice with ectopic expression of FGF-18, thus demonstrating a unique role of FGF-18 signaling in the chondrogeneic process to induce new cartilage formation or expand cartilage growth.

[0019] One embodiment of the present invention relates to a pharmaceutical composition comprising FGF-18 protein in an amount effective to induce cartilage formation.

[0020] Another embodiment of the present invention relates to a method for inducing cartilage formation in an affected area of a patient requiring such treatment comprising the step of administering to the affected area a pharmaceutical composition containing an amount of FGF-18 protein effective to induce cartilage formation in the affected area.

[0021] Another embodiment of the present invention relates to an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein.

[0022] Another embodiment of the present invention relates to a method of

expressing FGF-18 protein in a cell *in vitro*, comprising the step of providing an expression vector comprising at least one nucleotide sequences encoding FGF-18 protein.

[0023] Another embodiment of the present invention relates to a method for treating a patient in need of cartilage regeneration, repair, reconstruction, *de novo* formation or other cartilage formation in an affected area of the patient, the method comprising the step of introducing to the affected area an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein to form cartilage in the cells.

[0024] Another embodiment of the present invention relates to a method for treating a patient in need of cartilage regeneration, repair, reconstruction, *de novo* formation or other cartilage formation in an affected area of the patient, the method comprising the step of introducing to the affected area FGF-18 protein in an amount effective to induce formation of cartilage.

[0025] Another embodiment of the present invention relates to a cell culture comprising cells in a medium capable of sustaining cell growth, the cells having introduced therein an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein to form cartilage in the cells.

[0026] Another embodiment of the present invention relates to a cell culture comprising: (a) cells capable of producing cartilage in the presence of FGF-18 protein; and (b) a medium capable of sustaining cell growth that contains an amount of FGF-18 protein to induce formation of cartilage.

[0027] Another embodiment of the present invention relates to a method for preparing a cell culture for inducing cartilage formation *in vitro* in cells in a medium capable of sustaining cell growth, the method comprising the step of introducing into the cells an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein.

[0028] Another embodiment of the present invention relates to a cell culture comprising: (a) a first group of cells in a medium capable of sustaining cell growth; and (b) a second of group of cells of a type different from the first group of cells and co-cultured therewith, the second group of cells having introduced therein an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein to induce formation of cartilage.

[0029] Another embodiment of the present invention relates to a for treating a patient in need of cartilage regeneration, repair, reconstruction, *de novo* formation or other cartilage formation in an affected area of the patient, the method comprising the step of administering

to the affected area FGF-18 protein in an amount effective to induce formation of cartilage in the affected area.

BRIEF DESCRIPTION OF DRAWINGS

[0030] Fig. 1 is an image of a Northern Blot analysis of transgene specific mouse FGF-18, total FGF-18, and ß-actin mRNAs assessed in fetal mouse lung at E16 and correlated with the presence and absence of the transgenes.

[0031] Fig. 2 is an image (original magnification X4) of the expression of FGF-18 perturbing lung histology after hematoxylin-eosin staining of lung tissue from control (A and C) and double transgenic pups (B, D) at E16 (A, B) and E19, (C, D).

[0032] Fig. 3 is an image of the edge of fetal lung from FGF-18 treated and control littermates.

[0033] Fig. 4 is an image (original magnification X10) of the effects of FGF-18 on TTF-1, proSP-C, SP-B, and CCSP after immunostaining for TTF-1, proSP-C and SP-B (taken at original magnification X20), and CCSP performed on lungs of wild type (WT) pups (A,C,E,G,I) or double transgenic pups (B,D,F,H,J) expressing FGF-18.

[0034] Fig. 5 is an image (original magnification X10) of lungs from FGF-18 expressing and control littermates (E19) immunostained for PECAM (A,B) and α smooth muscle actin (C,D,E,F).

[0035] Fig. 6 is an image of lung tissue from control (A) and FGF-18 expressing (B) littermates.

[0036] Fig. 7 is an image of lungs dissected from fetal mice and stained with alcian blue, and tissue digested with KOH prior to photography under a dissecting microscope, of affected (A) and control (B) mice treated with doxycycline from E6.

[0037] Fig. 8 is an image (original magnification X4) of hematoxylin-eosin staining of cartilage rings in wild type (A) and FGF-18 expressing mice at E16 (B).

[0038] Fig. 9 is an image (original magnification X4) of *in situ* hybridization performed with radiolabeled FGF-18 antisense (A,B) and sense probes (B,C) on sections of fetal mouse tissue from wild type mice on E18.5 (upper panels).

BRIEF DESCRIPTION OF SEQUENCE LISTINGS

[0039] SEQ ID NO:1 shows the nucleotide sequence of the cDNA for FGF-18 (house mouse).

[0040] SEQ ID NO:2 shows the amino acid sequence for FGF-18 protein (house mouse).

[0041] SEQ ID NO:3 shows the nucleotide sequence of the cDNA for FGF-18 (human).

[0042] SEQ ID NO:4 shows the amino acid sequence for FGF-18 protein (human).

DETAILED DESCRIPTON OF THE INVENTION

1. Definitions

[0043] As used herein, the term "pluripotent:" refers to the ability of a cell to differentiate into a wide variety of mature cell types. The term "multipotent" refers to the ability of a cell to differentiate into a more limited variety of mature cell types than that of a pluripotent cell.

[0044] As used herein, the term "stem cells" refers to any pluripotent cell type, and can be derived from embryonic or adult tissues.

[0045] As used herein, the term "autologous" refers to use of a recipient's or patient's own cells or tissues as a source for transplantation. By contrast, the term "heterologous" refers to cells or tissues transplanted from another human or species.

[0046] As used herein, the term "somatic" refers to cells other than those arising from germ cells, germ cells being the cells that produce gametes, i.e., spermatozoa and ova.

[0047] As used herein, the term "cartilaginous tissue" refers to chondrocytes, and tissue which is formed by chondrocytes, which demonstrate the histological and compositional characteristics of cartilage.

[0048] As used herein, the term "gene" means a sequence of genetic material (e.g., DNA and RNA) that carries the information encoding a polypeptide (e.g., protein).

[0049] Unless otherwise indicated herein, the term "polypeptide" means a protein, polypeptide or peptide.

[0050] As used herein, the term "vector" means an agent comprising, consisting essentially of, or consisting of a DNA or RNA molecule capable of introducing a nucleic acid sequence(s) into a cell, resulting in the expression of the nucleic acid sequence(s) in the cell. Examples include but are not limited to a modified plasmid or virus that carries a gene or cDNA into a suitable host cell and there directs expression or synthesis of the encoded polypeptide.

[0051] As used herein, the terms "FGF-18" and "FGF-18 protein" refer to Fibroblast

Growth Factor-18, the polypeptide (FGF-18 protein) that is capable of inducing formation of cartilage. FGF-18 protein (house mouse) has the amino acid sequence shown in SEQ ID NO:2 and GenBank accession number AB004639, while FGF-18 protein (human) has the amino acid sequence shown in SEQ. ID NO:4 and GenBank accession number AB007422. See Ohbayashi et al, "Structure and Expression of the mRNA Encoding a Novel Fibroblast Growth Factor, FGF-18," *J. Biol. Chem.*, (1998) 273(29):18161-64, which is incorporated by reference. The amino acid sequence of SEQ ID NO:2 is identical to that of GenBank file AF075291, which includes untranslated flanking nucleotide sequences lying 5' and 3' to the coding sequences. See Hu et al., "FGF-18, A Novel Member of the Fibroblast Growth Factor Family, Stimulates Hepatic and Intestinal Proliferation," *Mol. Cell. Biol.*, (1998) 18(10):6063-74, which is incorporated by reference.

[0052] As used herein, the terms "nucleotide sequence(s) encoding FGF-18" or "nucleotide sequence(s) encoding FGF-18 protein" refer to the portion of the FGF-18 gene that codes for the polypeptide amino acid sequences of the bioactive FGF-18 protein. The cDNA of FGF-18 (house mouse) has the nucleotide sequence shown in SEQ ID NO:1 and has the GenBank accession number NM_008005 and AF075291, while the cDNA of FGF-18 (human) has the nucleotide sequence shown in SEQ ID NO:3 and has the GenBank accession numbers NM_033649, NM_003862 and AF075292. See Ohbayashi et al, "Structure and Expression of the mRNA Encoding a Novel Fibroblast Growth Factor, FGF-18," *J. Biol. Chem.*, (1998) 273(29):18161-64, which is incorporated by reference. GenBank sequence file NM_008005 comprises the coding sequence for FGF-18 that is identical to AB004639, as well as 5' and 3' flanking sequences. See Hu et al., "FGF-18, A Novel Member of the Fibroblast Growth Factor Family, Stimulates Hepatic and Intestinal Proliferation," *Mol. Cell. Biol.*, (1998) 18(10):6063-74, which is incorporated by reference.

[0053] As used herein, the term "gene promoter" refers to that portion of the nucleotide sequence of the gene that regulates, controls or otherwise modulates (e.g., stimulates or suppresses) the expression by the particular gene. For example, a gene promoter can enhance transcription and/or translation of the gene, thus increasing the mRNA levels transcribed from that gene.

[0054] As used herein, the term "regulatory elements" refer to sequences required for processing of mRNA transcribed from a transgene or other mammalian gene. Such elements are well know to those with ordinary skill in the art, and include but are not limited to enhancers, introns, and poly-A sequences.

[0055] As used herein, the term "gene expression" refers to steps at the level of the DNA molecule that lead to the production of a gene polypeptide product. Thus, gene expression is to be understood herein as culminating in the production of the polypeptide encoded by the gene so expressed.

[0056] As used herein, the term "mammal" refers to humans and nonhuman mammals, including primates (e.g., humans, monkeys, baboons, macaques), dogs, cats, rabbits rats, gerbils, hamsters, mice, horses, cows, goats, and other species commonly known as mammals.

[0057] As used herein, the term "intended recipient" is intended to include patients or individuals in need of therapeutic restoration or generation of cartilage or cartilaginous tissue(s).

[0058] As used herein, the term "affected area of the patient" means the area of the patient (e.g., joint, nose, ear, eye, trachea, etc.) requiring treatment to induce cartilage formation, or the area proximate thereto that is capable of inducing cartilage formation if treated according to the present invention

[0059] As used herein, the term "comprising" means various agents, compositions, compounds, genes, polypeptides, components, steps and the like can be conjointly employed in the present invention. Accordingly, the term "comprising" encompasses the more restrictive terms "consisting essentially of" and "consisting of."

[0060] As used herein, the terms "therapeutic agent," "pharmaceutical," and "drug" are used interchangeably to refer to a pharmacological composition, formulation or compound, including those useful for administration to cells or tissues *in vitro* or *in vivo* to induce cartilage formation.

[0061] As used herein, the term "pharmaceutically acceptable salt" means non-toxic salts of compounds (which are generally prepared by reacting the free acid with a suitable organic or inorganic base) and include, but are not limited to, the acetate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, calcium, camsylate, carbonate, chloride, clavulanate, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynapthoate, iodide, isothionate, lactate, lactobionate, laurate, malate, maleate, mandlate, mesylate, methylbromide, methylnitrate, methylsulfate, mucate, napsylate, nitrate, oleate, oxalate, pamaote, palmitate, panthothenate, phosphate, diphospate, polygalacturonate, salicylate, stearate, subacetate, succinate, tannate, tartrate,

teoclate, tosylate, triethiodide, and valerate salts, as well as mixtures of these salts.

[0062] All amounts, parts, ratios and percentages used herein are by weight unless otherwise specified.

2. Methods for Inducing or Stimulating Cartilage Formation

[0063] One aspect of the present invention are methods for inducing or stimulating cartilage formation through the use of FGF-18. In these methods, FGF-18 can be administered *in vivo* to induce or promote the formation of therapeutically useful cartilage-producing cells and/or cartilage in a patient requiring such therapy, can be administered to cells or tissues for *in vitro* induction of such cartilage formation with subsequent introduction (e.g., by implanting, transplanting, or other transfer method) of the cartilage or cartilage producing cells formed into the affected area (e.g., joint, nose, ear, eye, trachea, etc.) of the patient requiring such therapy, or can be administered *in vitro* to cells or tissues capable of inducing such cartilage formation with subsequent introduction (e.g., by implanting, transplanting, or other transfer method) of the induced cells or tissues *in situ* into the affected area (e.g., joint, nose, ear, eye, trachea, etc.) patients requiring such therapy for subsequent formation of the inducted cartilage.

[0064] In carrying out the various embodiments of this method, FGF-18 protein is administered to cells capable of committing to a chondrocyte cell fate. Suitable cells of this type include but are not limited to immature or mature chondrocytes, mesenchymal cells, adult stem cells such as neural stem cells and bone marrow stromal cells, embryonic stem cells, fibroblasts, myoblasts, osteoblasts, and other pluripotent or multipotent cell types. Other somatic cells that can be induced to de-differentiate to a multipotent cell type can also be used. Administration of FGF-18 protein to these cell types promotes differentiation and maturation of the chondrocytic phenotype. The chondrocytes thus formed can synthesize and secrete collagenous matrix proteins to form cartilage structures *in vitro* or *in vivo*.

[0065] In one embodiment of this method, *in vitro* formation of cartilage is obtained by administration of FGF-18 protein to cultured immature or mature chondrocytes, mesenchymal cells, adult stem cells such as neural stem cells and bone marrow stromal cells, embryonic stem cells, fibroblasts, myoblasts, osteoblasts, and other pluripotent or multipotent cell types. Scaffold or matrix material can be used to promote formation of cartilage structures of the desired shapes or sizes. Natural scaffold or matrix can be provided by cartilage excised from donor sites in the intended recipient or patient, or from a heterologous donor source, including other human donors, or from animal sources, such as pig, dog,

rodent, or other suitable mammalian species. Artificial sources for scaffold or matrix material include but are not limited to biodegradable and non-biodegradable synthetic polymer-based biomaterials such as polyglycolic acid (PGA) and poly-L-lactic acid (PLLA) or their composite mixtures, polytetrafluoroethylene, polyethylmethacrylate, or hydroxyapatite/Dacron composites. See Suh et al, "Application of Chitosan-based Polysaccharide Biomaterials in Cartilage Tissue Engineering: A Review," *Biomaterials*, (2000) 21:2589-98; Freed et al, *J. Biomed. Mater. Res.*, (1994) 28:891-99; Woo et al, *Plastic. Reconstr. Surg.*, (1994) 94(2):233-37; Athanasiou et al, *Biomaterials*, (1996) 17:93-102, Reisses et al, *Transactions of the Orthopaedics Research Society*, (New Orleans, LA, 1994); Messner et al, *Biomaterials*, (1993) 14:513-21, which are incorporated by reference. Scaffold or matrix material can be placed in the culture vessel containing cells that may be induced to differentiate into chondrocytes.

[0066] FGF-18 protein can be administered to the cells *in vivo* in solution added to the culture media, or can be impregnated in or otherwise bound to the scaffold or matrix material, or attached to beads or other particles that can be placed in the culture vessel, or in or on the scaffold or matrix material. Alternatively, FGF-18 protein can be produced by cells in culture, either through endogenous expression of an FGF-18 gene, or by acquisition of an FGF-18 gene resulting from gene transfer to the cultured cells. Gene transfer can be achieved by introduction of a functional transgene comprising promoter and regulatory elements and FGF-18 encoding sequences contained within a viral vector, including but not limited to lentiviral, adenoviral, adeno-associated or other viral sequences capable of transducing mammalian cells. Gene transfer can also be mediated by liposomal or other carriers or mechanical delivery systems.

[0067] In another embodiment of this method, cells are grown in sheets rather than on a scaffold or matrix material. Similar modes of FGF-18 protein administration can be provided to cultured immature or mature chondrocytes, mesenchymal cells, adult stem cells such as neural stem cells and bone marrow stromal cells, embryonic stem cells, fibroblasts, myoblasts, osteoblasts, and other pluripotent or multipotent cell types will promote differentiation of mature chondrocytes. The cells can grow and differentiate in confluent sheets of cartilage-producing cells. The sheets of differentiated cells thus produced can then be harvested and introduced to the affected area where cartilage formation is desired in the intended recipient. Administration of FGF-18 protein can also be included as part of a therapy to maintain the chondrocytic phenotype of the transplanted cells. Localized

administration can be achieved using beads or other particles coated or impregnated with FGF-18 protein, or with FGF-18 protein covalently or otherwise functionally bound or linked to the beads or particles. These beads or particles can be made of biodegradable or non-biodegradable materials, including synthetic polymer-based biomaterials include PGA, PLLA or their composite mixtures, polytetrafluoroethylene, polyethylmethacrylate, or hydroxyapatite/Dacron composites. Non-corrosive metals, including but not limited to stainless steel, or precious metals, including but not limited to gold, can also be used as beads or other particles to which FGF-18 protein is covalently or otherwise bound or linked. Metered administration of FGF-18 protein can also be directed by implantation of a mini-pump such that the outflow allows application of FGF-18 protein to the area where cartilage formation is desired.

[0068] Other embodiments of this method include *in vivo* formation of cartilage or cartilaginous tissues, either *in situ* in desired affected areas within the patient in need of such therapy or treatment, in ectopic sites within the intended recipient and subsequently introduced (e.g., transplanted) to the desired site, or in a human or non-human host/donor. *In vivo* formation of cartilage can be induced *in situ* where cartilage growth is desired by delivery of FGF-18 protein to cells capable of producing collagenous matrix materials that form cartilage. In cases where endogenous cells are capable of producing collagenous matrix materials, FGF-18 protein can be administered in the location where cartilage formation is desired. Localized administration can be achieved using beads or other particles coated or impregnated with FGF-18 protein, or with FGF-18 protein covalently or otherwise functionally bound or linked to the beads or particles, as previous described. In another embodiment where a scaffold or supporting matrix is desired to direct formation of cartilage structures, FGF-18 protein can be coated or impregnated on or in the scaffold or matrix, or covalently or otherwise bound or linked to the scaffold or matrix. Alternatively, FGF-18 protein can administered via expression of an FGF-18 gene acquired by gene transfer (as previously described) to endogenous cells surrounding the area of the scaffold or matrix implant.

[0069] In another embodiment of this method where endogenous cells are not capable of producing collagenous matrix materials, administration of FGF-18 protein can be accompanied by transplantation of immature or mature chondrocytes, mesenchymal cells, adult stem cells such as neural stem cells and bone marrow stromal cells, embryonic stem cells, fibroblasts, myoblasts, osteoblasts, or other pluripotent or multipotent cell types into the

desired site of cartilage formation. These cells can be obtained from the intended recipient or patient as an autologous donation; or obtained from a heterologous donor source, including other human donors; cultured cells such as immature or mature chondrocytes, mesenchymal cells, adult stem cells such as neural stem cells and bone marrow stromal cells, embryonic stem cells, fibroblasts, myoblasts, osteoblasts, and other pluripotent or multipotent cell types; or from animal sources, such as pig, dog, rodent, or other suitable mammalian species.

[0070] In yet another embodiment of this method, FGF-18 protein can be administered via expression of an FGF-18 transgene acquired by cells through gene transfer, with gene transfer (as previously described) occurring prior to transplantation. Cells transduced with the FGF-18 gene-containing vector are selectively cultivated and transplanted to the site where cartilage formation is desired. Suitable cells include any transducible non-transformed mammalian cell type, but primary cells harvested from the intended recipient or patient are preferred. Transducible cell types include, but are not limited to, cells such as fibroblast, osteoblasts, bone marrow stromal cells, neural stem cells, or myoblasts. Similar cell types from a heterologous donor can also be used.

[0071] When *in vivo* formation of cartilage cannot be induced *in situ,* or where *de novo* growth of cartilage is desired, cartilaginous tissues or structures can be grown in ectopic regions and transplanted to the desired site(s). Such ectopic regions can be located within the intended recipient or patient, and are most likely to be regions of endogenous cartilage. Examples of such regions include but are not limited to the cartilaginous regions of the sternum, ribs, pelvis, ears, and nose. Scaffold or matrix material can be implanted in suitable regions of naturally occurring cartilage, and FGF-18 protein is administered to recruit local chondrocytes to produce cartilage structures of the desired shape or size. Natural scaffold or matrix materials can be provided by cartilage excised from ectopic donor sites in the intended recipient or patient, or from a heterologous donor source, including other human donors, or from animal sources, such as pig, dog, rodent, or other suitable mammalian species. Artificial sources for scaffold or matrix material can include but is not limited to biodegradable and non-biodegradable synthetic polymer-based biomaterials previously described, with the FGF-18 protein being coated on, impregnated within, or covalently or otherwise bound or linked to the scaffold or matrix material to induce formation of cartilage or cartilaginous structures of the desired size and shape, as previously described. Metered administration of FGF-18 protein can also be directed by implantation of a mini-pump, as previously described, or via expression of an FGF-18 gene acquired by gene transfer, as previously described. The

cartilage or cartilaginous structures thus formed can subsequently be surgically excised and implanted or transplanted to the desired site in the intended recipient or patient. Alternatively, these cartilage or cartilaginous structures can be produced in a similar manner in a host/donor other than the intended recipient or patient, including other humans, or animals such as pig, dog, rodent, or other suitable mammalian species. The cartilage or cartilaginous structures thus formed can subsequently be surgically excised and transplanted to the desired site in the intended recipient or patient.

[0072] Embodiments of this method can use FGF-18 protein to induce cartilaginous tissue or other tissue formation in circumstances where such tissue is not normally formed, and has application in the healing of cartilage, for example articular cartilage tears, deformities and other cartilage defects in the affected area of patients (human or otherwise). FGF-18 protein can be employed for prophylactic use in preventing damage to cartilaginous tissue, as well as use in the improved fixation of cartilage to bone or other tissues, and in repairing defects to cartilage tissue. *De novo* cartilaginous tissue formation can be induced by FGF-18 protein for the repair of congenital, trauma induced, or other cartilage defects, and is also useful in surgery for attachment or repair of cartilage. FGF-18 protein can also be useful in the treatment of arthritis and other cartilage diseases, as well as other indications wherein it is desirable to heal or regenerate cartilage tissue. Such indications include, without limitation, regeneration or repair of injuries to the articular cartilage, e.g., cartilage of the joint such as the knee, ankle, shoulder or elbow.

[0073] In addition to articular cartilage, FGF-18 protein can be suitable for treatment of other translucent cartilages such as costal cartilages (e.g., cartilage connecting the ribs and sternum), in the septum of the nose, and particularly cartilage tissue formation relating to the conducting airways such as the trachea, bronchi, lung and larynx. For example, FGF-18 protein can be used to induce new cartilage formation or expand cartilage growth the tracheal-bronchial rings of the conducting airways, as well as the larynx. FGF-18 protein can also be useful in the treatment of problems other affected areas of the patient such as the ear and the cornea.

3. Pharmaceutical Compositions and Packaged Drugs

[0074] FGF-18 protein can be formulated as a pharmaceutical composition or packaged drug for use in the previously described methods. The pharmaceutical compositions include a therapeutically effective amount of FGF-18 protein, and optionally a pharmaceutically acceptable carrier. The packaged drug includes the FGF-18 protein,

optionally a pharmaceutically acceptable carrier, and instructions for administering or using the drug. The set of instructions can be written or printed on sheet of paper, can be on the packaging associated with the packaged drug, can be in the form of electronic media or software (e.g., floppy disk or CD ROM disk) that can be loaded, installed (directly or by downloading from a remote site such as via a LAN, WAN or the Internet), or otherwise can be read by a computer, personal digital assistant (PDA) or other electronic device, or any other suitable method for providing instructions on how to administer the to treat the subject.

[0075] Although FGF-18 protein can be administered alone, it is preferably administered as part of a pharmaceutical formulation. Such formulations can include pharmaceutically acceptable carriers known to those skilled in the art, as well as other therapeutic agents. It will also be appreciated that the formulations of the present invention can be administered in various pharmaceutically acceptable forms, e.g., as pharmaceutically acceptable salts.

[0076] Appropriate dosages FGF-18 protein administered in accordance with the present invention will depend on the desired location of formation of cartilage and the amount of cartilage needed, and can also vary from patient to patient. Determining an acceptable or optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the dose and treatment of the present invention. For a dose to be "therapeutically effective," it must have the desired effect, i.e., induce the formation or expansion of cartilage or cartilaginous tissues in the desired location

[0077] In addition to FGF-18 protein, pharmaceutical formulations of the present invention can also comprise additional compounds and/or compositions that will also aid in differentiation of chondrocytes and/or induction of cartilage formation. The ratio of FGF-18 protein to any additional compounds will depend upon the dose desired of each of the individual compounds. Preferably, the composition will be administered as a pharmaceutically-acceptable aqueous solution wherein the pharmaceutical formulation comprises: (1) from about 0.001% to about 10% FGF-18 protein; (2) from about 10% to about 99% of a pharmaceutically-acceptable carrier; and (3) from about 0.001% to about 10% of any additional compound(s).

[0078] Administration of FGF-18, with or without a pharmaceutically acceptable carrier(s) and/or additional compound(s), can be by any suitable route including coating on, impregnating within, or covalently or otherwise binding or linking to the scaffold or matrix material to induce formation of cartilage or cartilaginous structures of the desired size and

shape. Metered administration of FGF-18 protein can also be directed by implantation of a mini-pump, as previously described, or via expression of an FGF-18 gene acquired by gene transfer, as previously described. Formulations suitable for minipump administration include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the body fluids of the intended recipient; and aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. The formulations can be in unit or multi-dose containers, for example, sealed ampules and vials, and may be lyophilized, requiring only the addition of the sterile liquid carrier such as water for injections immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described.

Examples

[0079] The following example illustrates the capability FGF-18 to induce cartilage formation in the conducting airways.

A. Experimental Procedures

1. Transgenic Mice

[0080] A permanent transgenic mouse line bearing the SP-C-rtTA transgene is established in FVB/N background after oocyte injection of a plasmid construct consisting of 3.7 kb of the human SP-C promoter, placed 5′ to the rtTA gene construct (27,28,31). Mouse FGF-18 cDNA is inserted between the $(teto)_7$CMV promoter and the 3′ untranslated region of the bovine growth hormone gene. See Clark et al, *Am. J. Physiol.*, (2001) 280,:L705-15. The rtTA and $(teto)_7$ constructs are provided by Dr. Herman Bujard, ZMBH-Heidelberg. See Gossen et al, *Proc. Natl. Acad. Sci. USA*, (1992) 89:5547-51. Offspring of all founders are screened by Southern blot or PCR analysis. Mice transmitting the $(teto)_7$CMV-FGF-18 transgene are bred to SP-C-rtTA mice. The transgenic SP-C-rtTA "activator" line used is stable for more than three years in the vivarium. Heterozygous and homozygous $(teto)_7$CMV-FGF-18 mice are viable and without observable abnormalities. Two separate target lines bearing the $(teto)_7$CMV-FGF-18 transgene (lines A and B) are chosen for breeding to SP-C-rtTA activator mice. Transmission of both transgenes followed typical Mendelian inheritance patterns. All mice are maintained in a pathogen free vivarium. Doxycycline (0.5 mg/ml) is administered in drinking water or in the food pellets (25 mg/g; Harlen Teklar, Madison, WI) for the described time periods. The drinking solution containing doxycycline is changed 3 times per week, while activity of the doxycycline is stable in the food pellet. See Perl et al,

Transgenic Res., (2002) 11:21-29.

2. RT-PCR

[0081] Tissues are homogenized in Tri-Zol (Life Technologies) and RNA is isolated according to the manufacturer's specifications. RNA is treated with DNAse prior to cDNA synthesis. Five µg RNA is reverse transcribed, then analyzed by PCR for murine FGF-18, and transgene specific FGF-18 and ß-actin mRNAs. Transgene specific primers for mouse FGF-18 are designed to the $(teto)_7$CMV-FGF-18 transcript, and used for amplification. Primer A is located in the CMV minimal promoter (5′ to 3′) AGA CGC CAT CCA CGC TGT TTTG; primer B in the FGF-18 cDNA (5′ to 3′) CAG GAC TTG AAT GTG CTT CCC ACTG. FGF-18 mRNA is compared to that amplified for ß-actin. FGF-18 mRNAs are also estimated using primers designed to amplify within the FGF-18 coding sequence using standard gel analysis of PCR products. FGF-18, FGF-10, SHH, BMP-4, and Sprouty-2 mRNAs are also determined by real time PCR of lung cDNA, after optimization of primers and conditions. Dams are placed on doxycycline throughout pregnancy, sacrificed on E15 and RNA extracted from the lungs of each pup. cDNA is prepared by reverse transcription and analyzed on the Smart Cycler® using primers to identify ß-actin, FGF-18, BMP-4, Sprouty-2 and FGF-10. All results are normalized to ß-actin.

3. Histology, Immunohistochemistry, and Electron Microscopy

[0082] To obtain fetal lung tissue, the fetuses are removed by hysterotomy after lethal injection of pentobarbital to the dam. The chest of fetal animals is opened and the tissue fixed with 4% paraformaldehyde at 4°C. Lungs from postnatal animals are inflation-fixed at 25 cm water pressure via a tracheal cannula with the same fixative. Tissue is fixed overnight, ished in PBS, dehydrated through a series of alcohols, and embedded in paraffin. Tissue sections are stained for SP-B, proSP-B, TTF-1, proSP-C, CCSP (Clara cell secretory protein), PECAM (peripheral endothelial cell adhesion molecules), α-smooth muscle actin, Foxj1, and procollagen II using methods described in Clark et al, *Am. J. Physiol.*, (2001) 280:L705-15 and Tichelaar et al, *J. Biol. Chem.*, (2000) 275:11858-64. Cartilage is stained with Alcian blue and residual tissue dissolved in KOH prior to photography. For electron microscopy, tissue is fixed, prepared, and evaluated, as previously described. See Clark et al, *Am. J. Physiol.*, (2001) 280:L705-15.

4. *In Situ* Hybridization

[0083] Expression of mouse FGF-18 mRNA is assessed by *in situ* hybridization using ^{35}S-labeled riboprobes for fetal and adult lungs, the latter after inflation fixation at 25 cm of

water pressure. See Clark et al, *Am. J. Physiol.*, (2001) 280:L705-15. Sense and antisense FGF-18 RNA probes are generated in PGEM32. Tissue is hybridized overnight at 50°C. Slides are coated with Kodak NTB2 emulsion, exposed for 7-14 days, and developed with Kodak D19. Whole mount *in situ* hybridization for mouse FGF-18, FGF-10, SHH, BMP-4, and Sprouty-2 are performed by digoxigenin labeled cDNA antisense and sense probes. Whole mount *in situ* hybridization is carried out on lungs of fetal day 12 embryos, whose dam had been on doxycycline throughout pregnancy. Anti-sense and sense probes are made from transcription vectors, using digoxigenin-UTP as label. After hybridization and ishing, anti-digoxigenin antibody coupled to alkaline phosphatase is adsorbed. The product is developed using BM purple alkaline phosphatase substrate.

B. Results

1. Generation of SP-C-rtTA and $(teto)_7$CMV-FGF-18 Transgenic Mice

[0084] In the absence of doxycycline, double transgenic SP-C-rtTA and $(teto)_7$CMV-FGF-18 mice (heterozygous for each transgene) are viable. Fetal and postnatal single transgenic mice are produced in ratios predicted by Mendelian inheritance. Lung morphology is normal in both single transgenic mice and in the double transgenic mice in the absence of doxycycline. Prior *in situ* hybridization and reporter gene analyses of the lungs from SP-C-CAT and SP-C-rtTA mice, demonstrate that transgenic mRNA is selectively expressed in peripheral respiratory epithelial cells in the lungs of fetal and adult mice. Expression of firefly luciferase with the SP-C-rtTA system is observed as early as E10 *in vivo*. See Perl et al, *Transgenic Res.*, (2002) 11:21-29. In adult SP-C-rtTA mice, rtTA mRNA is selectively expressed in peripheral conducting airways and type II epithelial cells. See Tichelaar et al, *J. Biol. Chem.*, (2000) 275:11858-64 and Perl et al, *Transgenic Res.*, (2002) 11:21-29. Two independent lines of $(teto)_7$CMV-FGF-18 target mice (lines A and B) are generated and mated to SP-C-rtTA mice. A similar morphologic phenotype is observed in the lungs of the independent $(teto)_7$CMV-FGF-18 lines after exposure to doxycycline. Subsequent studies utilized $(teto)_7$CMV-FGF-18 line "A."

2. Conditional Expression of FGF-18 mRNA

[0085] Transgene specific FGF-18 mRNA is assessed by RT-PCR in lungs of young adult mice, with and without addition of 0.5 mg/ml doxycycline in the drinking water. In adult double transgenic SP-C-rtTA x $(teto)_7$CMV-FGF-18 mice, FGF-18 mRNA is detectable at low levels in the absence of doxycycline, representing some "leak" in the absence of doxycycline, but is induced after oral doxycycline with the SP-C-rtTA mice. See Clark et al,

Am. J. Physiol., (2001) 280:L705-15 and Tichelaar et al, *J. Biol. Chem.*, (2000) 275:11858-64. Exposure of adult double transgenic mice to doxycycline did not alter lung morphology. In pups obtained from dams treated with doxycycline, FGF-18 mRNA is detected in fetal SP-C-rtTA x $(teto)_7$CMV-FGF-18 double transgenic mice, but is not readily detected in single transgenic animals. See lane A (wild type), lane B (single transgenic $(teto)_7$-FGF-18), lanes C-E (double transgenic), and lane F (double transgenic with no RT) of the Northern blot image in Fig. 1. Transgenic FGF-18 mRNA is not detected in other major organs of double transgenic mice, including liver, spleen, kidney, and brain, typical of the specificity of the SP-C promoter element, which is generally active only in respiratory epithelial cells in the lung. See Glasser et al, *Am. J. Physiol.*, (1991) 261:L349-56. Exogenous FGF-18 mRNA is detected in testes of a double transgenic mouse on doxycycline, albeit at extremely low levels compared to that in lung.

4. Effects of FGF-18 on the Fetal Lung

[0086] When dams are exposed on E6 and maintained on doxycycline during pregnancy, the percent survival of the offspring decreased to 50% of expected Mendelian numbers in double transgenic mice, consistent with lethality of the transgene. The structure of lungs from double transgenic SP-C-rtTA x $(teto)_7$CMV-FGF-18 offspring is assessed at E16-19. See Fig. 2 where marked abnormalities in the conducting airways and lung parenchyma are noted in FGF-18 expressing mice, while wild type embryos are unaffected. Arrows mark abnormal, large caliber, peripheral airways with features typical of the more proximal regions of normal lung.. Without doxycycline, lung histology of double transgenic mice is generally indistinguishable from normal. In the presence of doxycycline, dramatic histologic abnormalities are observed in the lungs from double transgenic mice on E16-19. Branching morphogenesis is disrupted. A marked increase in length and caliber of conducting airways is observed. Decreased branching of peripheral airways with a marked reduction in peripheral saccules is consistently noted. The abnormal histology is not observed on E12.5, but is readily apparent at E16 and thereafter. Abnormally large airways and cysts are readily apparent in the FGF-18 expressing mice by direct visualization of the lung periphery. See Fig. 3 showing dissected lungs (E17) from double transgenic and control pups after exposure to doxycycline from E6 as photographed under a dissecting microscope, with arrows indicating dilated saccules.

[0087] Later in gestation, E18-19, stage specific sacculation of peripheral acinar buds and alveoli are lacking in the FGF-18 expressing mice. See Fig. 2. Elongated conducting

airways of disordered caliber extended to the lung periphery. Atypical branching of bronchioles and distal bronchiolar-acinar tubules is observed, and normal acinar ducts and alveoli are markedly decreased or absent. The lung mesenchyme is thickened, containing few acinar tubules. Pulmonary blood vessels are prominent with abnormally large lumens, and alveolar capillaries are lacking. The residual peripheral airway saccules are not dilated and the normal alveolar structures of the newborn lung are lacking. Large, abnormal bronchial-like tubules, are observed throughout the lungs and many extended to the pleural surfaces. While similar abnormalities are observed in FGF-18 expressing fetal mice, variability in the extent of the histologic abnormalities is observed even in double transgenic mice from the same litter, suggesting that the timing, extent, or levels of transgene expression may influence the severity of the phenotype. Similar lung abnormalities are observed from litters capable of producing offspring with one copy or two copies of the CMV-FGF-18 transgene and in both A and B $(teto)_7$FGF-18 lines.

4. Aberrant Morphology and Differentiation of Epithelial Cells Lining the Lung Tubules

[0088] The peripheral conducting tubules in FGF-18 expressing mice at E16-19 are lined by a relatively homogenous population of columnar and cuboidal epithelial cells cilia. These abnormal airway epithelial cells stained intensely and homogeneously for TTF-1, reflecting a lack of terminal differentiation, and failure to form squamous cells (type I) in the periphery at E16-19 and newborns. See Fig. 4. ProSP-C and SP-B are detected at relatively low levels throughout the abnormal epithelium, consistent with the lack of both type II and squamous type I cell differentiation at E16 and 19. Atypical staining of surfactant proteins is observed in both basalar and apical regions of the cells while staining is detected in apical regions of type II cells in the normal lung. Abnormal clumps of cells staining for CCSP (Clara cell secretory protein) are observed in the elongated dilated respiratory bronchioles. However, CCSP is excluded from the most peripheral regions of lung tubules at E16 and 19 (see Fig. 4), as it is in the alveolar regions of the normal lung. The atypical cells lining lung tubules did not express Foxj1, a marker of ciliated cells in normal conducting airways, data not shown.

5. FGF-18 Altered Differentiation and Morphology of the Pulmonary Mesenchyme

[0089] Pulmonary vascular development is perturbed as indicated by the abnormalities of PECAM staining in the pulmonary mesenchyme of the FGF-18 expressing

mice. See Fig. 5. Extensive blood vessel development is noted in the abnormal mesenchyme surrounding the sparse, relatively small acinar tubules. Atypical pulmonary blood vessels in the periphery often had markedly enlarged lumenal diameter. See Fig. 5. α-Smooth muscle actin (α-SMA) staining, normally abundant in proximal, conducting airways and excluded from the alveolar region, is observed surrounding the aberrant airways in the lung periphery, being detected at sites that normally lack α-SMA staining in control littermates. See Fig. 5.

6. Ultrastructure of Fetal Lung from FGF-18 Expressing Mice

[0090] Ultrastructural analysis of lung tissue from FGF-18 expressing mice at E16 and 18 is consistent with observations at the light microscopic level. Abnormally large peripheral airways are lined by an atypical columnar epithelium. A relatively homogenous population of immature epithelial cells is observed in the peripheral tubules. Most of the terminal airspaces are lined by cuboidal or columnar cells and few squamous (type I) cells are observed. Epithelial cells are rich in glycogen, often lacked microvilli, and contained few lipid inclusions. See Fig. 6. Tubular myelin is not observed in the airways. Some of the atypical cells contained basal bodies, the latter typical of developing tracheal-bronchial ciliated cells. In lungs from control littermates, developing pre-type II cells are cuboidal and contained putative lamellar bodies. Tubular myelin is occasionally observed. At E18, squamous type I cells lined the most peripheral saccules in control pups. In FGF-18 expressing mice, the abnormal lung mesenchyme is poorly organized and contained abnormal blood vessels surrounded by prominent smooth muscle cells. Abnormal spaces are observed between the stromal cells in the pulmonary mesenchyme.

7. FGF-18 Perturbed Tracheal-Bronchial Cartilage

[0091] Consistent abnormalities in tracheal and bronchial cartilage are observed in the mice expressing FGF-18. Disordered size and shape of tracheal-bronchial cartilage rings and marked expansion of bronchial cartilage is consistently observed. See Fig. 7. Histologic analysis and procollagen II immunostaining demonstrates the abnormal bronchial cartilage that is readily detectable as early as E15-16.5, but not at E12.5. See Fig. 8

8. Effects of FGF-18 on FGF-10, BMP-4, and Sprouty-2 mRNAs

[0092] In whole mount *in situ* hybridization on lungs from E12 embryos, distribution and intensity of FGF-10, Sprouty-2, and BMP-4 mRNAs are not altered in FGF-18 expressing mice. Light cycler® analysis for BMP-4, Sprouty-2, and FGF-10 mRNAs confirmed the lack of effect of FGF-18 on these mRNAs.

9. *In situ* Hybridization for Endogenous FGF-18 mRNA

[0093] *In situ* hybridization with radiolabeled mouse FGF-18 antisense RNA demonstrated that FGF-18 mRNA is expressed at high concentrations in stromal cells surrounding tracheal-bronchial cartilage rings, in tissue surrounding laryngeal cartilage, and in the mesenchyme of the normal fetal lung from E12.5-18. See Fig. 9.

C. Discussion

[0094] FGF-18 mRNA is conditionally expressed in respiratory epithelial cells of the lungs of fetal and postnatal mice. FGF-18 has little effect on the postnatal lung. However, lung morphogenesis is perturbed by expression of FGF-18 in the fetal lung. FGF-18 increases the length, caliber and disrupted branching of peripheral conducting airways, abnormal cytodifferentiation of epithelial cells lining the bronchial like lung tubules, blocks sacculation and alveolarization in late gestation, perturbs the organization of lung mesenchyme, increasing the extent and size of vascular structure and inhibiting capillary invasion of the lining epithelium, and induces cartilage in the periphery of main bronchi. Taken together, FGF-18 influences various aspects of proximal-distal programming of the lung, enhancing elements of the conducting airways and inhibiting those of the lung periphery.

1. Effects of FGF-18 on Lung Structure

[0095] FGF-18 produces a homogenous cuboidal-columnar epithelium that lacked features characteristic of normal, peripheral tubules. The atypical columnar epithelial cells are rich in glycogen, and lacked other features typical of type II cells. Squamous cell differentiation is inhibited. On the other hand, some aspects of proximal epithelial cell differentiation are not apparent in the abnormal epithelial cells. Neither cilia, Foxj1 nor CCSP staining are observed in most of the atypical epithelial cells in the peripheral lesions induced by FGF-18. Likewise, FGF-18 does not alter the levels or sites of expression of FGF-10, BMP-4, and Sprouty-2 mRNAs, suggesting that the effects of FGF-18 on lung morphology are not mediated via these pathways.

[0096] The sites and levels of ectopic expression of FGF-18 may influence the observed morphological effects of the FGF-18 transgene. Since FGF-18 is expressed in epithelial cells and not in mesenchymal cells, as in wild type mice, bioavailability of the ligand or accessibility of the ligands to FGF receptors, may be distinct in the transgenic mice. *In situ* hybridization for the endogenous FGF-18 mRNA confirms its expression in the pulmonary mesenchyme and demonstrated its distribution surrounding forming cartilage rings in the trachea and bronchi. This site of expression is consistent with a potential role for FGF-

18 in cartilage formation.

2. Increased Cartilage Formation

[0097] Expression of FGF-18 perturbs cartilage ring morphology in the trachea, and expands cartilaginous tissue in peripheral regions of main bronchi. The presence of endogenous FGF-18 mRNA surrounding normal cartilage rings in the developing trachea is also consistent with the role for FGF-18 and FGF-R signaling in tracheal-bronchial cartilage morphogenesis. Ectopic cartilage is not seen on E12.5, but is readily apparent at E16. Abnormalities in cartilage are not observed when FGF-18 is expressed postnatally. The shape and contiguity of cartilaginous rings are perturbed at normal sites of tracheal-bronchial cartilage formation; however, large amounts of cartilage formed in the distal bronchi of the FGF-18 expressing mice. These findings support the concept that FGF-18 altered proliferation of chondrocytes or prechondrocytes at critical times during morphogenesis. The abnormal cartilaginous tissue stained intensely for collagen type II, an early marker of cartilage differentiation.

[0098] Abnormalities in differentiation of the epithelial cells lining the conducting airways of the FGF-18 transgenic mice included atypical columnar morphology, the lack of saccular-alveolar differentiation, and is associated with a striking inhibition of type I and type II epithelial cell differentiation. Squamous type I cells and capillary invasion of peripheral tubules are lacking. Homogenous staining for TTF-1, proSP-C, and SP-B in peripheral epithelial cells and inhibition of differentiation of type I cells seen at the ultrastructural level are consistent with a failure of terminal differentiation of the peripheral lung parenchyma. However, characteristics of the abnormal epithelial cells are not consistent with a transformation of peripheral cells to a proximal epithelial cell type. While some of these abnormal cells contained basal bodies, usually associated with ciliated cells in proximal regions of the lung, the atypical cells in the most peripheral lesions did not express CCSP or contain numerous cilia, markers of normal mouse conducting airways. See Wert et al, *Dev. Biol.*, (1993) 156:426-43 and Tichelaar et al, *J. Histochem. Cytochem.*, (1999) 47:823-31. SP-B and proSP-C are localized within the atypical columnar cells in both basal and apical regions of the cells, a pattern not seen in normal alveolar type II cells, wherein these proteins are normally concentrated in apical membranes. Thus, changes in epithelial differentiation do not represent a complete proximalization of the peripheral respiratory epithelium, the abnormal cells sharing features of immature proximal and peripheral respiratory epithelial cells.

D. Conclusions

[0099] This experiment and resultant findings demonstrate that increased expression of FGF-18 markedly increases the length and caliber of conducting airways, and altered branching of the bronchial tree in peripheral regions of the fetal lung. Formation of acinar and alveolar elements that are normally lined by cuboidal and squamous epithelial cells is inhibited by FGF-18. Increased caliber of peripheral pulmonary blood vessels and lack of alveolar capillaries are consistent with enhancement of features of proximal lung structures and inhibition of alveolarization. FGF-18 induces ectopic cartilage and altered blood vessel formation and induced α-smooth muscle actin, consistent with the enhancement of development of proximal rather than peripheral regions of the lung. Effects of FGF-18 are limited to the fetal lung, and are not observed when the transgene is activated postnatally, supporting the concept that FGF-18 influences cell proliferation, differentiation or migration earlier in morphogenesis. FGF-18 has unique effects on lung formation, preferentially shifting some developmental and morphogenetic programs of blood vessels, cartilage, and airways towards proximal programs.

[0100] While specific embodiments of the present invention have been described, it will be apparent to those skilled in the art that various modifications thereto can be made without departing from the spirit and scope of the present invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising FGF-18 protein in an amount effective to induce cartilage formation.

2. A method for inducing cartilage formation in an affected area of a patient requiring such treatment comprising the step of administering to the affected area a pharmaceutical composition containing an amount of FGF-18 protein effective to induce cartilage formation in the affected area.

3. An expression vector comprising at least one nucleotide sequence encoding FGF-18 protein.

4. A method of expressing FGF-18 protein in a cell *in vitro*, comprising the step of providing an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein.

5. A method for treating a patient in need of cartilage formation in an affected area of the patient, the method comprising the step of introducing to the affected area an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein to induce cartilage formation in the cells.

6. The method of claim 5 wherein the affected area is a conducting airway.

7. The method of claim 6 wherein the conducting airway is at least one of the trachea, bronchi, lung and larynx.

8. A method for treating a patient in need of cartilage formation in an affected area of the patient, the method comprising the step of introducing to the affected area FGF-18 protein in an amount effective to induce cartilage formation.

9. The method of claim 8 wherein the affected area is a conducting airway.

10. The method of claim 9 wherein the conducting airway is at least one of the trachea, bronchi, lung and larynx.

11. A cell culture comprising cells in a medium capable of sustaining cell growth, the cells having introduced therein an expression vector comprising at least one nucleotide sequence encoding FGF-18 protein.

12. A cell culture comprising: (a) cells capable of producing cartilage in the presence of FGF-18 protein; and (b) a medium capable of sustaining cell growth that contains an effective amount of FGF-18 protein to induce cartilage formation in the cells.

13. A cell culture comprising: (a) a first group of cells in a medium capable of sustaining cell growth; and (b) a second of group of cells of a type different from the first group of cells and co-cultured therewith, the second group of cells having introduced therein an expression vector comprising nucleotide sequences encoding FGF-18 protein to induce formation of cartilage.

14. A method for preparing a cell culture comprising cells capable of inducing cartilage formation *in vitro* in a medium capable of sustaining cell growth, the method comprising the step of introducing into the cells an expression vector comprising a coding sequence for encoding FGF-18 protein.

15. A method for treating a patient in need of cartilage formation in an affected area of the patient, the method comprising the step of administering to the affected area FGF-18 protein in an amount effective to form cartilage in the affected area.

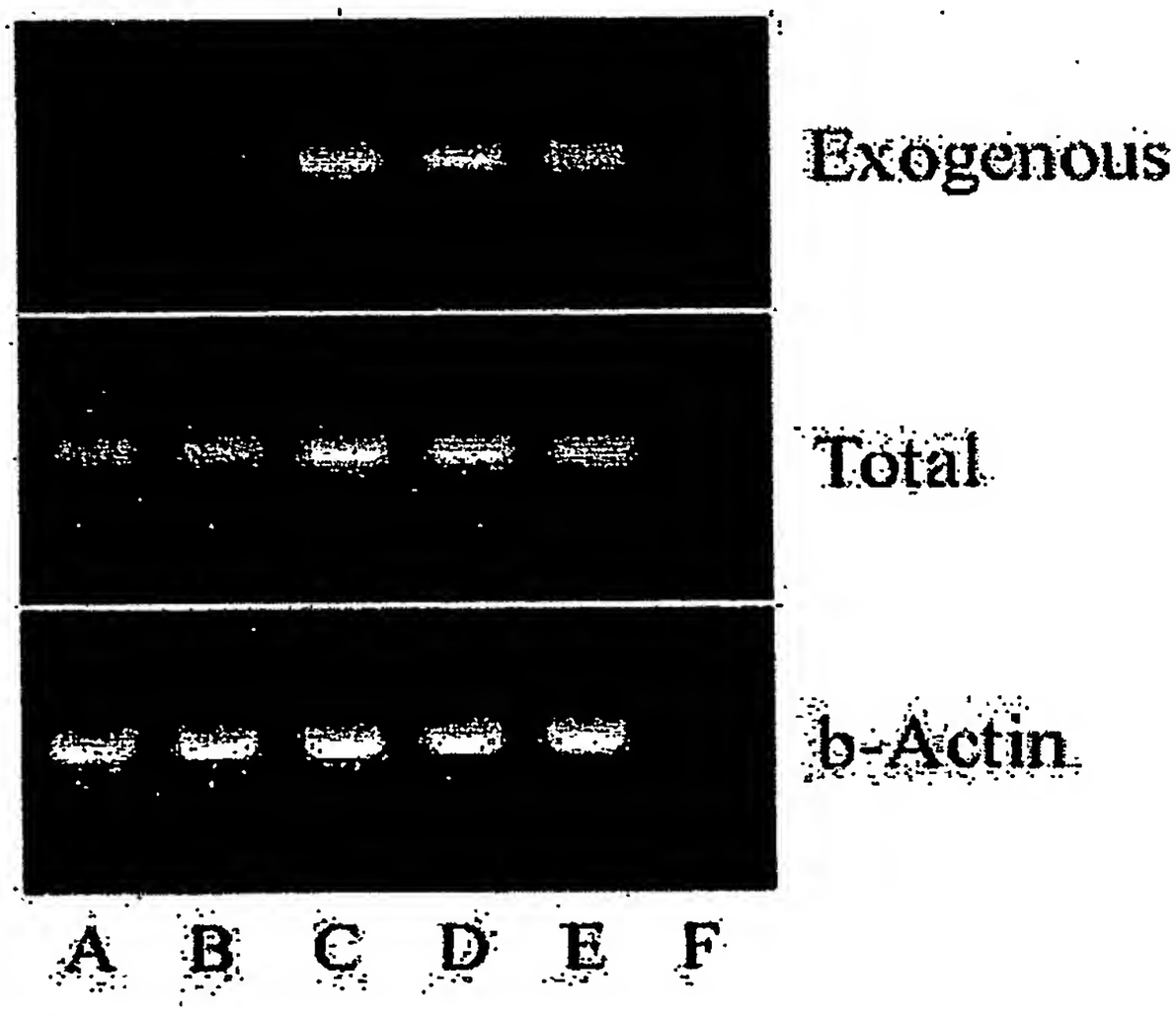
16. The method of claim 15 wherein the affected area is a conducting airway.

17. The method of claim 16 wherein the conducting airway is at least one of the trachea, bronchi, lung and larynx.

USE OF FGF-18 PROTEIN AND ITS RESPECTIVE ENCODING NUCLEOTIDE SEQUENCES TO INDUCE CARTILAGE FORMATION

ABSTRACT OF THE DISCLOSURE

[1001] The use of fibroblast growth factor (FGF)-18 protein, and the respective nucleotide sequences encoding this protein, particularly for inducing cartilage formation, particularly for the purpose of generating, repairing, reconstructing, or *de novo* formation of, cartilaginous tissue. Therapies for which FGF-18 are useful include repair and reconstruction of various tissues in conducting airways such as the trachea, bronchi, lung and larynx caused by, for example, tracheal-bronchial abnormalities, tracheal-laryngo or bronchial malaria. Other therapies for which FGF-18 would be useful include other cartilaginous tissues, such as those of joint and skeletal tissue caused by, for example, arthritis and meniscus abnormalities in joints.



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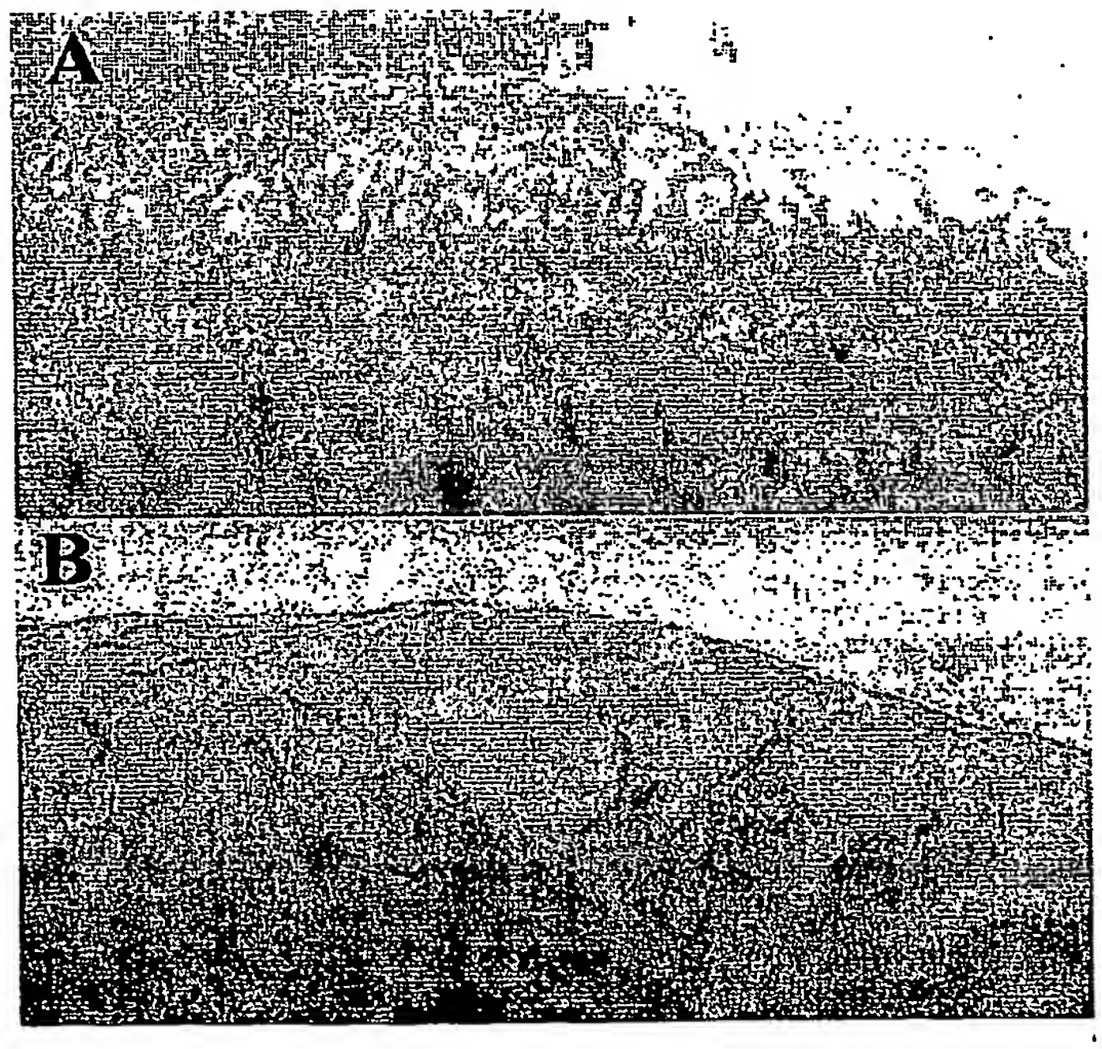

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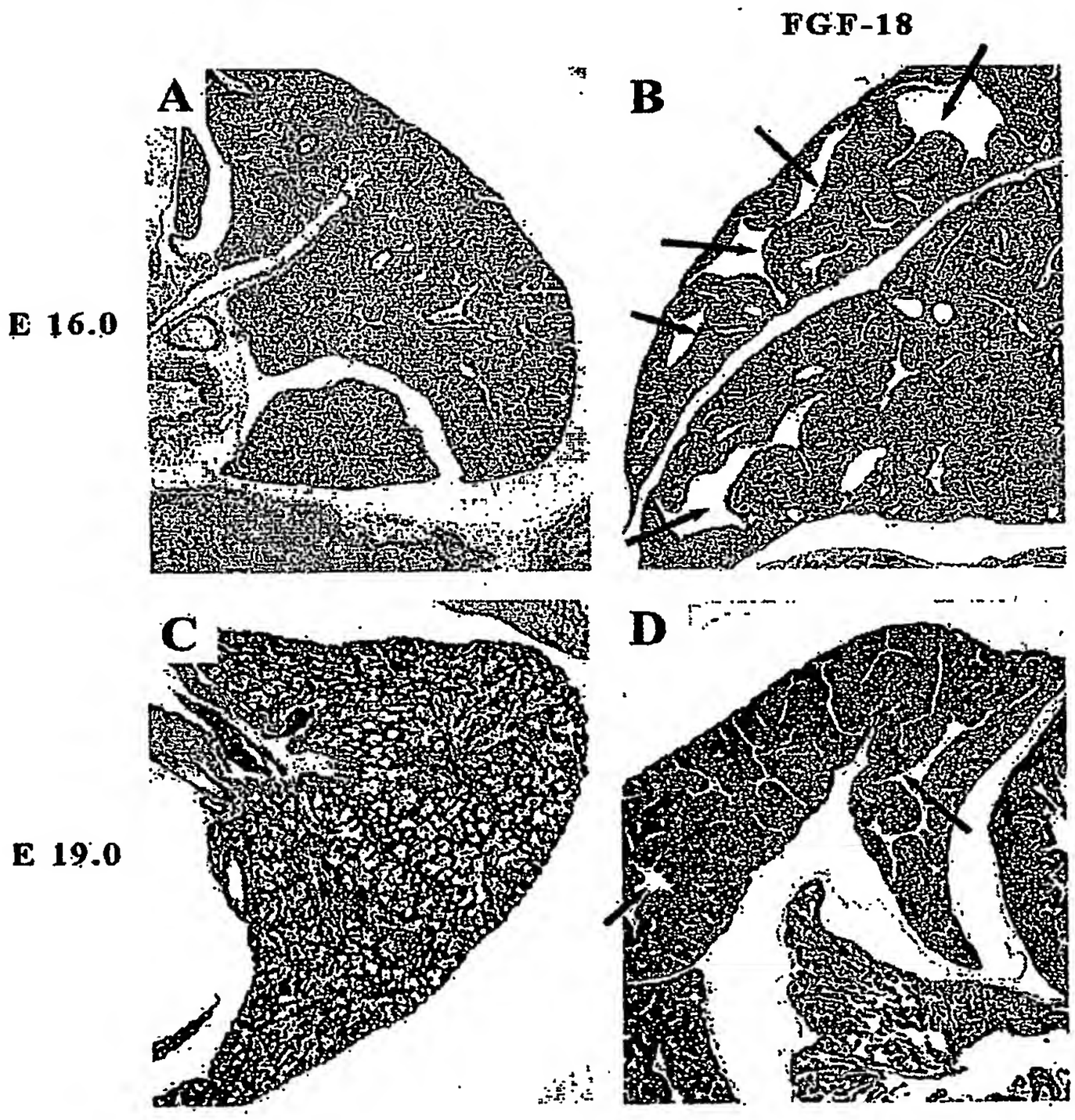

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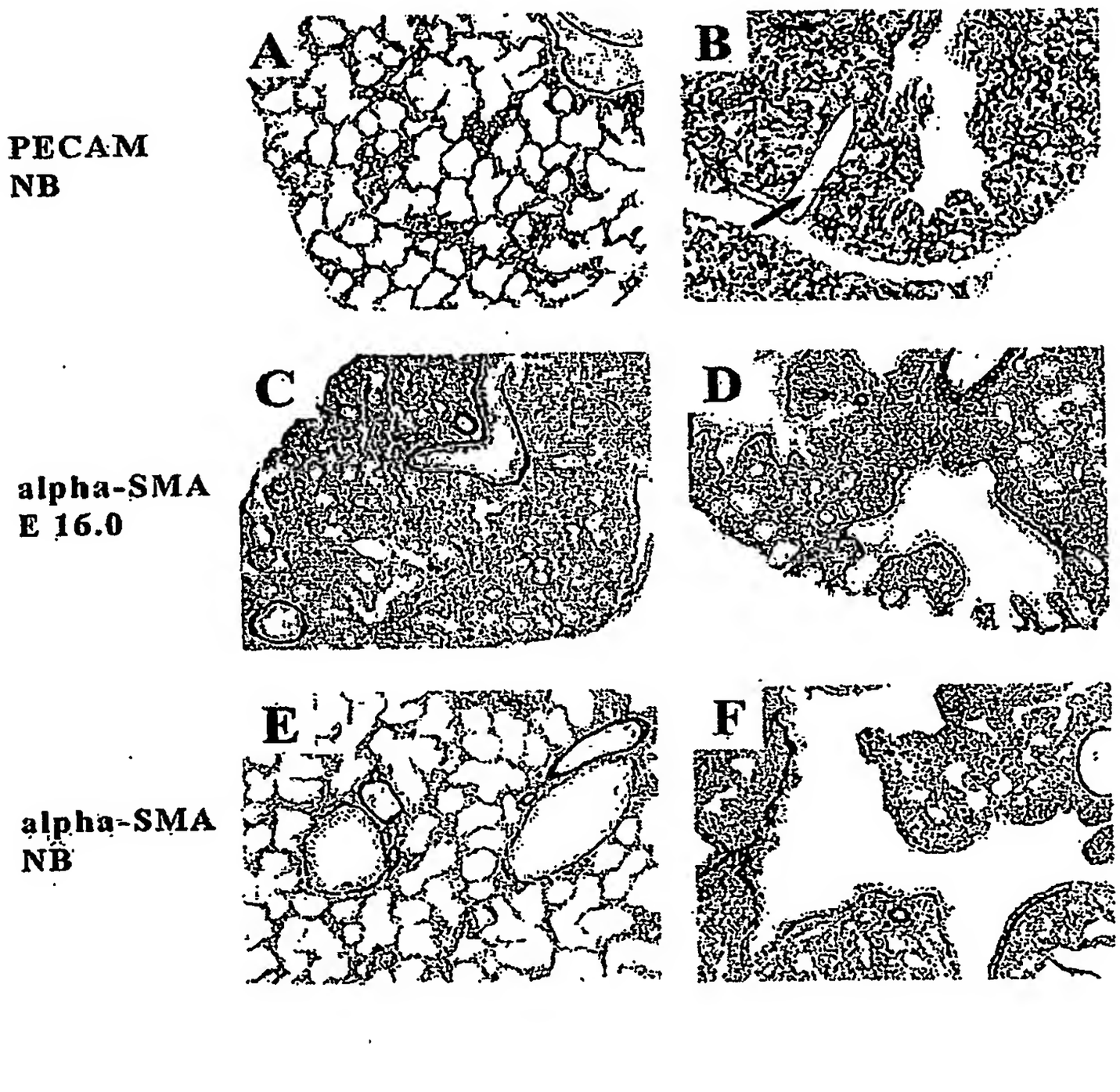

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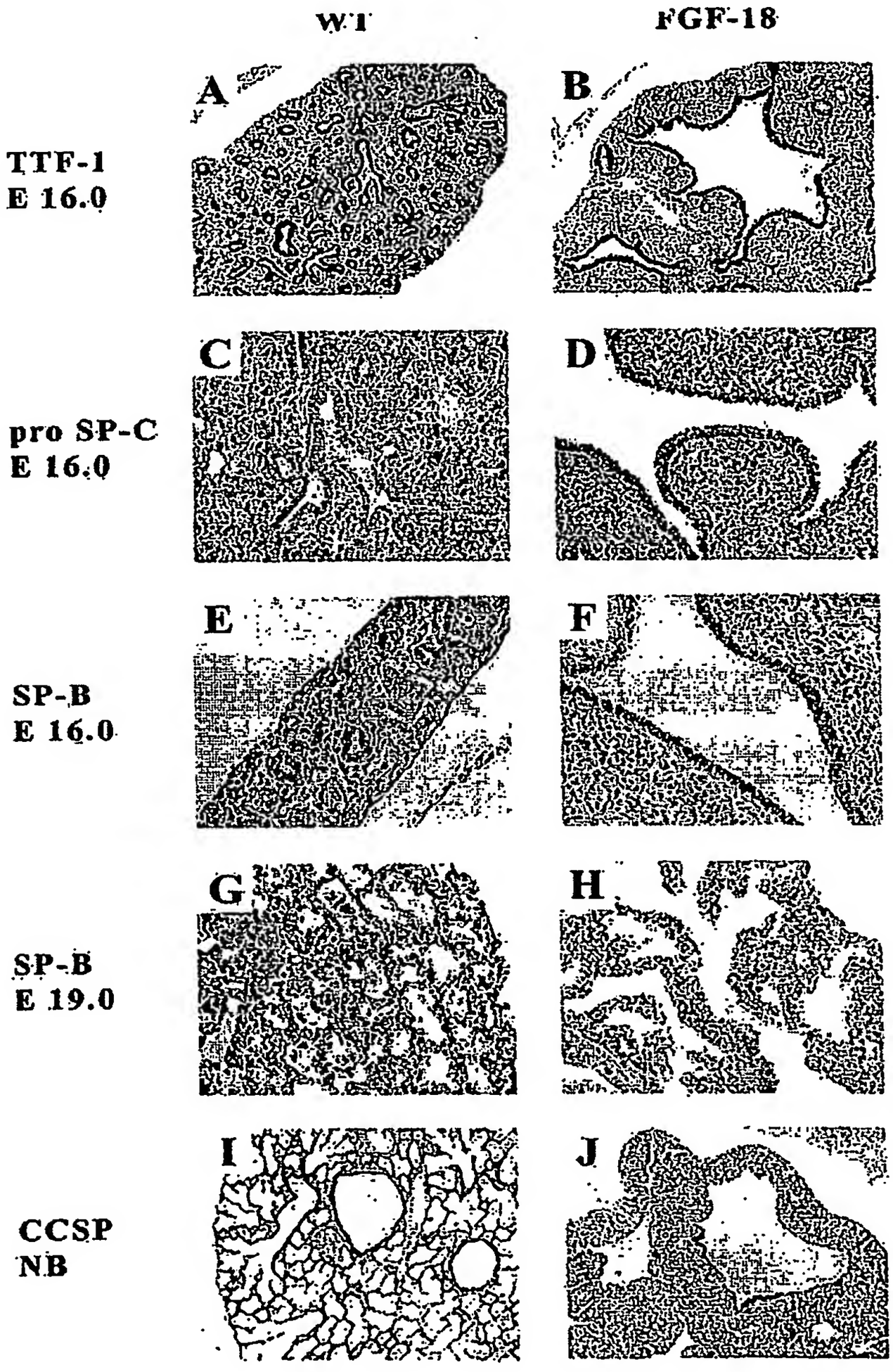
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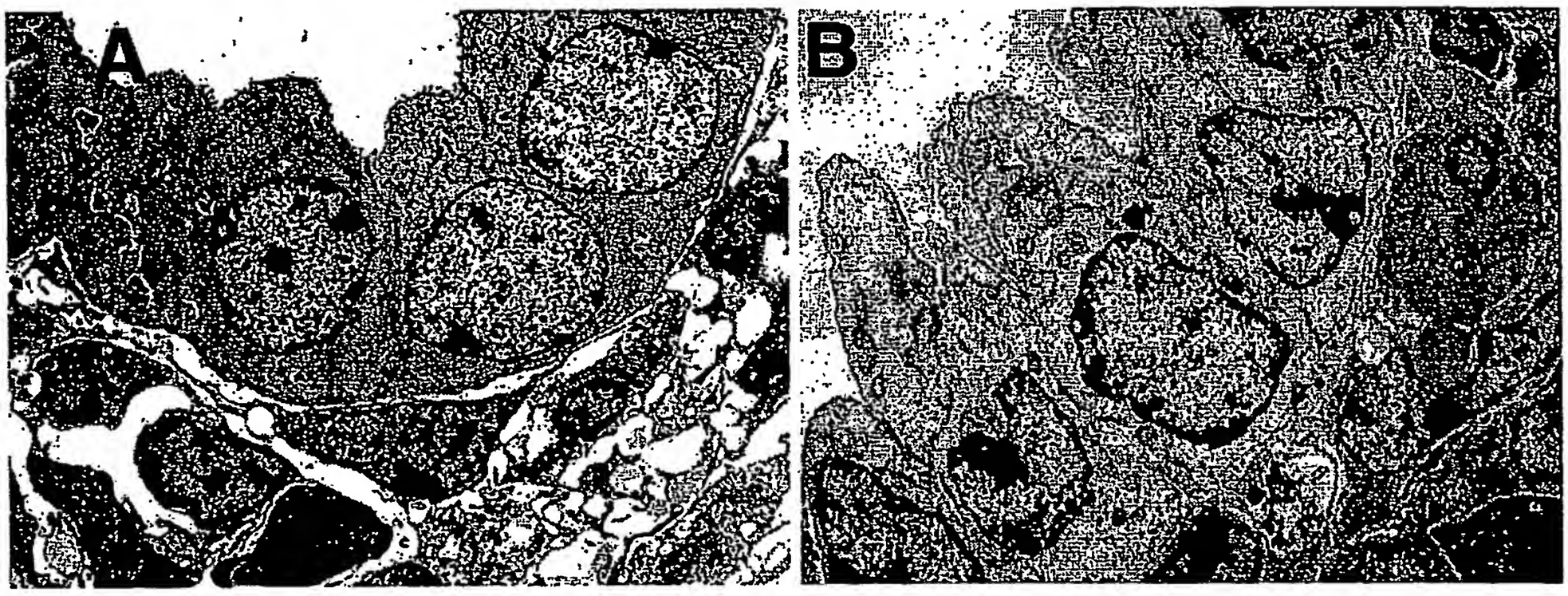
FIG. 5



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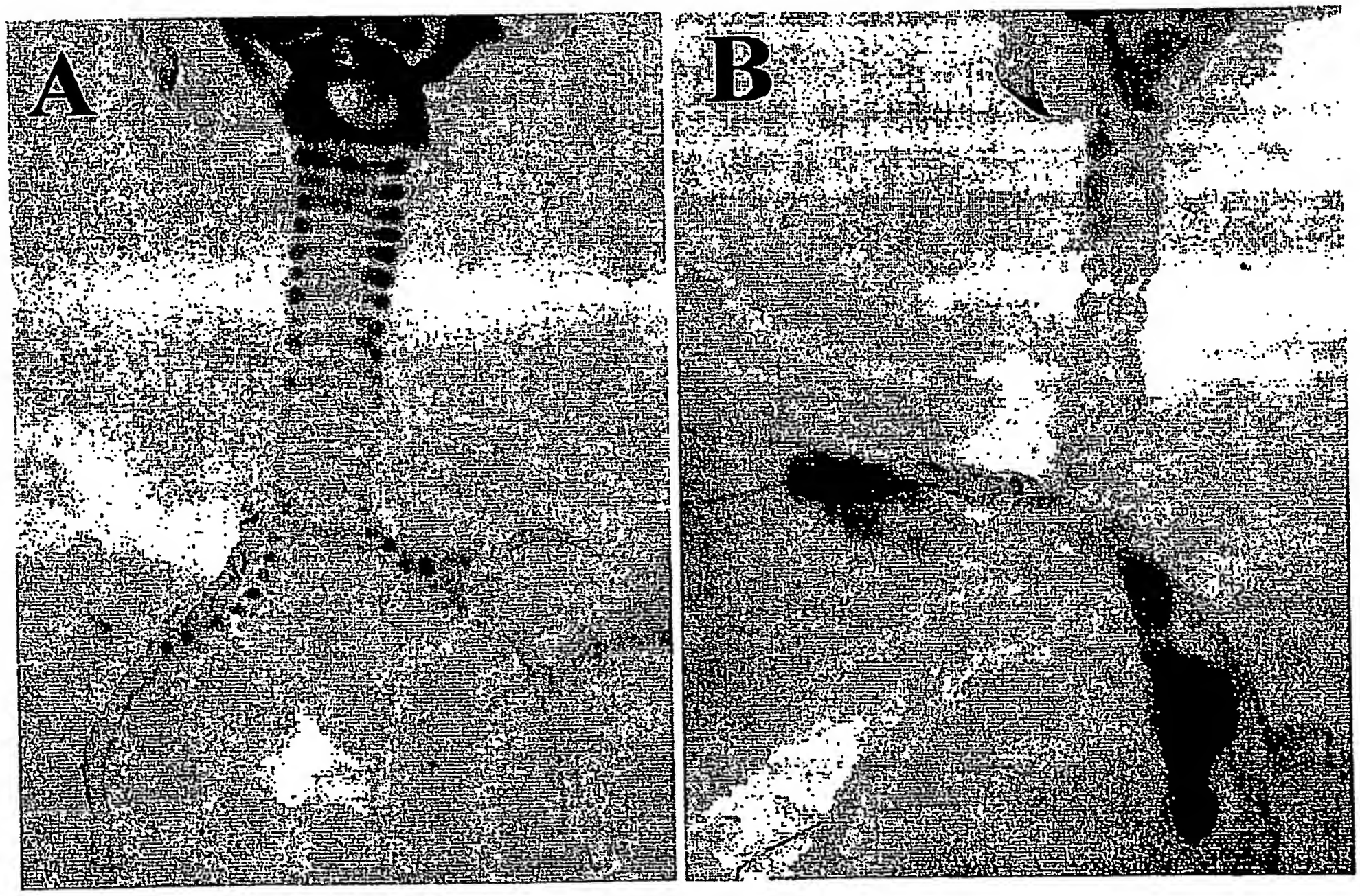

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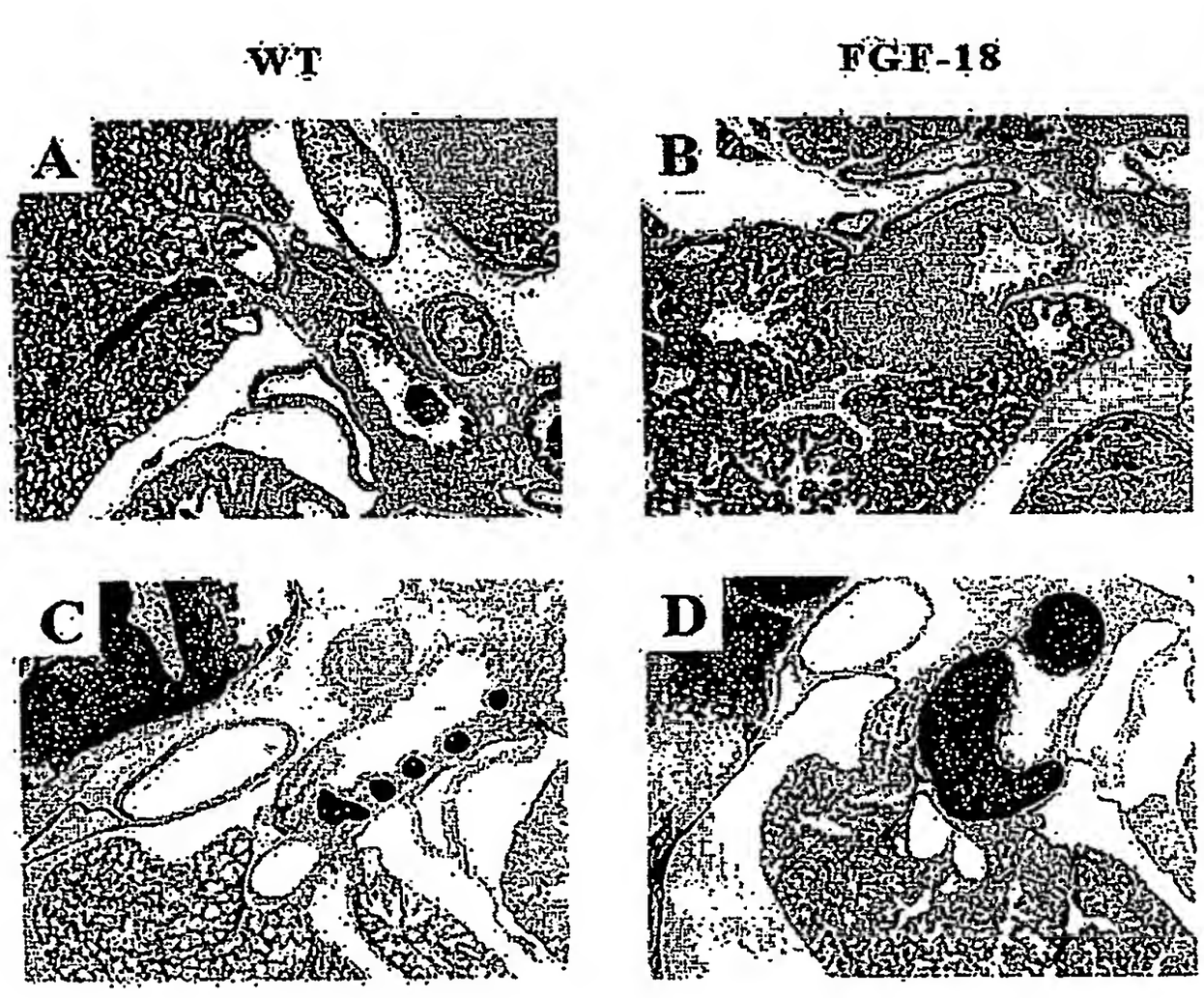

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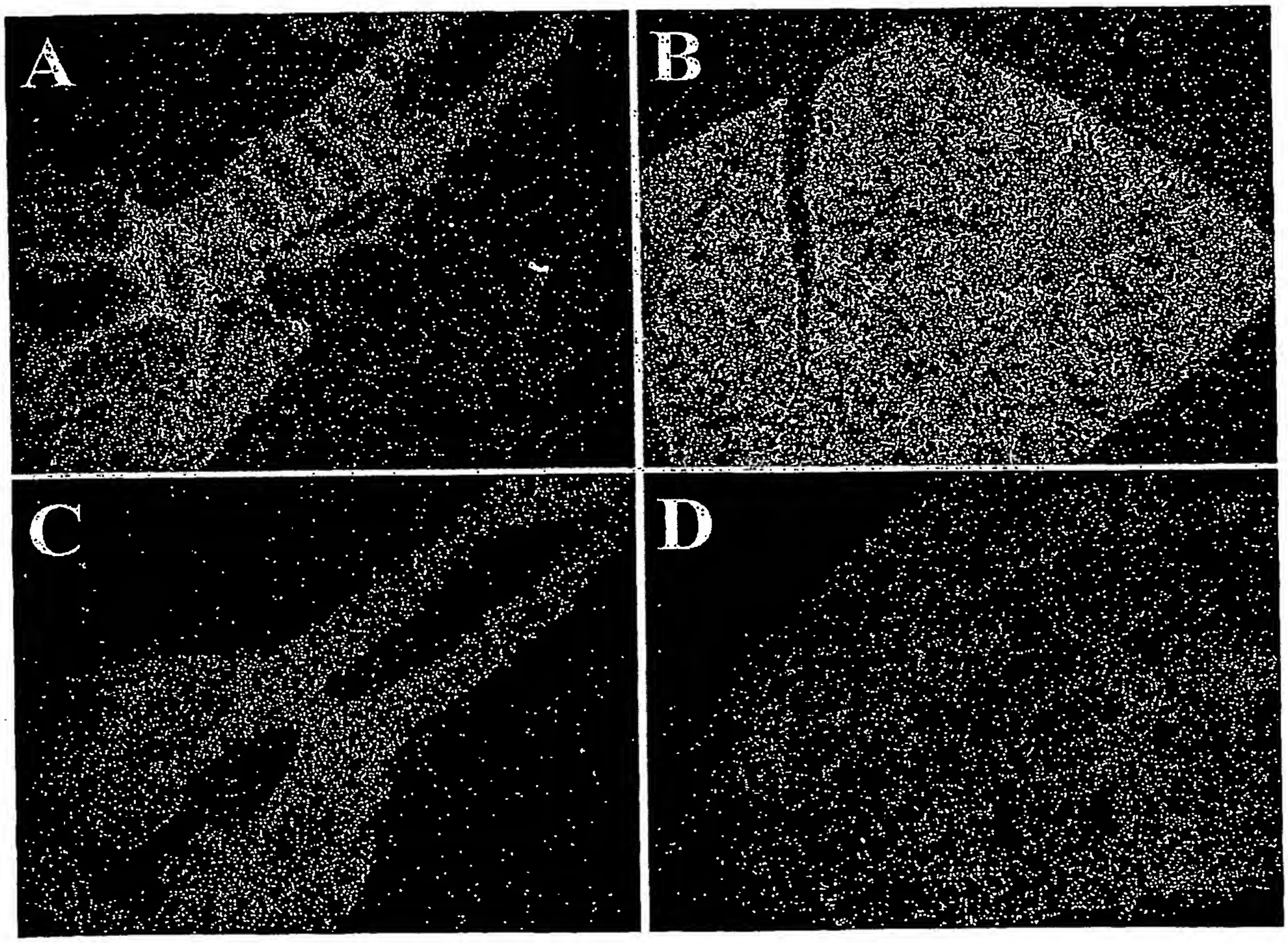

FIG. 9

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